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Cleavage behavior of calicheamicin γ^1 and calicheamicin T

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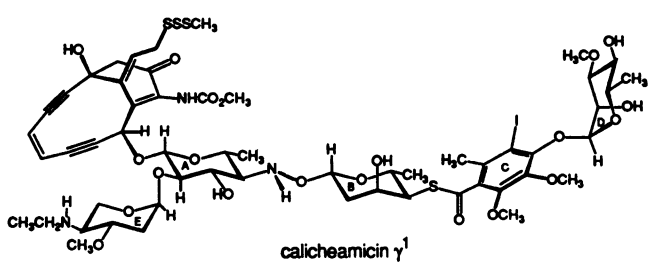
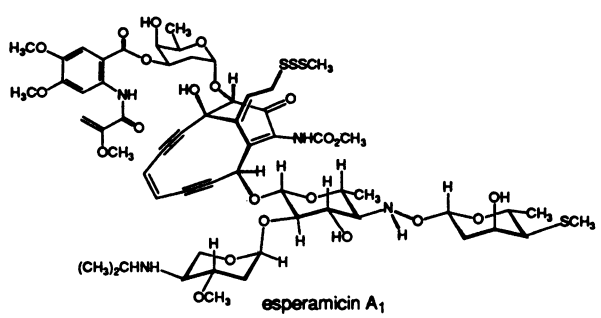
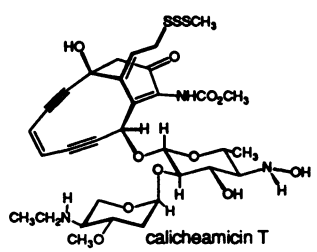
ABSTRACT **Calicheamicin γ^1 is a potent antitumor antibiotic that cleaves DNA with a high degree of specificity; there is much interest in the recognition process. We have investigated the DNA-cleaving properties of calicheamicin T, a truncated derivative of calicheamicin. We show that calicheamicin T cleaves DNA in a double-stranded fashion, indicating that the first two sugars are sufficient to facilitate binding of the aglycone in the minor groove. However, calicheamicin T cleaves DNA nonselectively. This result suggests that cyclization kinetics do not determine the cleavage specificity of the intact drug. Instead, cleavage specificity probably reflects binding specificity. Because of the recognition sites reported in the original cleavage paper, calicheamicin has been assumed to recognize oligopyrimidine DNA sequences containing G·C base pairs. We show here that calicheamicin also cuts efficiently at A·T tracts, sometimes in preference to G·C-rich homopyrimidine tracts. Crystallographic data and experiments with chemical probes indicate that DNA sequences including G·C base pairs have significantly different local conformations from DNA sequences containing several (four or more) sequential A·T base pairs. This difference makes it unlikely that calicheamicin simply senses inherent groove conformation and suggests that there is some degree of "induced fit." The ability to recognize both A·T- and G·C-rich oligopyrimidine sequences with a high degree of specificity makes calicheamicin an unusual minor-groove binder.**

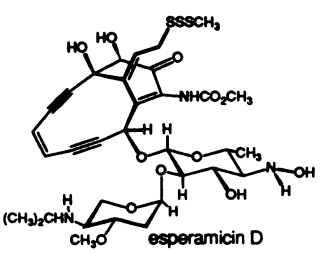
Calicheamicin γ^1 and esperamicin A_1 are potent antitumor antibiotics that contain diyne-ene functional groups (1–7). Their antitumor activity is apparently due to their ability to damage DNA. Upon treatment with a thiol cofactor, the diyne-ene moieties rearrange to form 1,4-benzenoid diradicals that abstract hydrogens from the sugar–phosphate backbone, initiating strand scission (8–10). Calicheamicin and esperamicin have attracted much attention recently because they cleave DNA site selectively. Calicheamicin, in particular, is highly site selective. Based on the cleavage-site preferences (TCCT, TCCC, TCCA, ACCT, TCCG, GCCT, CTCT, and TCTC) reported by Zein *et al.* (8), calicheamicin is generally considered selective for cytosine-containing oligopyrimidine tracts. It has been suggested that the selectivity for sequences including G·C base pairs is due to specific interactions between the exocyclic guanine amino groups in the minor groove and functional groups on calicheamicin (11). However, positions of the guanines in the reported recognition sites are variable, arguing against sequence-specific interactions. It has alternatively been suggested that the G·C selectivity reflects the conformational requirements of binding (12, 13). G·C-rich regions of DNA have wide minor grooves and, perhaps, other distinct structural features that allow calicheamicin to fit. However, no experiments that shed any light on this issue have been reported.





There have been a number of proposals as to how calicheamicin recognizes particular sites. Zein *et al.* (12) reported that the rhamnose sugar (D ring) and the ethylamino sugar (E ring) on calicheamicin can be removed without affecting cleavage specificity. It was proposed that the carbohydrate-aryl tail is a nonspecific binding element that helps target the drug to the minor groove. Cleavage selectivity was attributed to a shape-selective interaction between the aglycone and a conformation of DNA unique to TCCT and closely related sequences. Results on esperamicin D





indicate that the aglycone plus first two sugars cleave DNA with the same selectivity as the parent compound (10). Because the aglycones of esperamicin and calicheamicin are almost identical, the esperamicin results appear to support the proposal of Zein *et al.* (12). However, Drak *et al.* (13) recently reported that the aglycone of calicheamicin cleaves DNA nonspecifically. Drak *et al.* have proposed that the carbohydrate tail determines cleavage specificity of calicheamicin by binding to the DNA and positioning the aglycone for cleavage.

We have made a calicheamicin derivative that lacks the B-C-D rings of the carbohydrate tail. This compound, calicheamicin T, is almost identical to esperamicin D, which

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cleaves DNA sequence selectively at oligopyrimidine tracts and 5′ TG and 5′ CG sites (10). We have compared the cleavage behavior of calicheamicin T to that of calicheamicin γ^1 on a pUC19 restriction fragment identical to that used by Sugiura *et al.* (10) in their studies on esperamicin D. We have found that calicheamicin T shows no significant site selectivity. In the course of this work we also found that calicheamicin γ^1 cleaves two TTTT sites in the pUC19 restriction fragment. Calicheamicin γ^1 even cleaves a TTTT site in preference to an adjacent TCCC site, even though TCCC was reported in the original cleavage paper to be a principal recognition site (8). These cleavage sites are unusual in that they do not contain any G·C base pairs. We think it interesting that calicheamicin cleaves DNA quite selectively and yet recognizes both A·T-rich and G·C-rich oligopyrimidine tracts depending on context. The traditional division of minor-groove binders into G·C-rich binders and A·T-rich binders evidently does not apply to calicheamicin γ^1.

MATERIALS AND METHODS

Drugs and Chemicals. Calicheamicin γ^1 was isolated from *Micromonospora echinospora* ssp. *calichensis*. Calicheamicin T was produced from calicheamicin γ^1 by refluxing 10 mg of calicheamicin γ^1 in 10 ml of wet acetone with 10 mol % pyridinium tosylate. Calicheamicin T was purified from the reaction mixture by HPLC on a semipreparative Vydac C_{18} column (10 × 250 nm, peptides and proteins, 5 μm) with a gradient of CH_3CN/H_2O, 0.1% trifluoroacetic acid. To ensure absolute purity for the cleavage experiments, purified calicheamicin T was subjected to two more runs through the HPLC column.

Restriction enzymes and pUC19 were purchased from Boehringer Mannheim. Netropsin was purchased from Serva Biochemicals. Klenow fragment, polynucleotide kinase, and radiochemicals were purchased from New England Nuclear/DuPont.

Preparation and Labeling of Restriction Fragments. For the 3′-end-labeled fragment, plasmid pUC19 was digested with *Acc* I and *Nde* I, and the small fragment [244 base pairs (bp)] was isolated from an agarose gel by electrophoresis onto a DEAE-cellulose membrane (14). The fragment was recovered from the membrane and then labeled at the *Acc* I end using [α-^{32}P]dCTP and Klenow fragment. The labeled DNA was precipitated twice and then redissolved in water.

For the 5′-end-labeled fragment, plasmid pUC19 was digested with *Acc* I, labeled at both 5′ ends with [γ-^{32}P]dATP and polynucleotide kinase, and then digested with *Nde* I. The small fragment was isolated from an agarose gel as above (14).

Cleavage of Supercoiled pBR322. Each 20-μl reaction mixture contained calicheamicin γ^1 (at concentrations from 0 mg/ml to 0.5 mg/ml) or calicheamicin T (at concentrations from 0 mg/ml to 50 mg/ml) and supercoiled pBR322 (50 mg/ml, or 1.0 μg total weight) in a Tris buffer (40 mM Tris·HCl/4 mM EDTA, pH 7.5) containing 10% (vol/vol) ethanol (calicheamicin is added in ethanol for solubility). Reactions were initiated by adding dithiothreitol (1.0 mM), and the samples were incubated at 37°C for 30 min. The samples were then mixed with loading buffer (14), and half of each sample was loaded directly on a 0.9% agarose gel. After electrophoresis, the gel was photographed on Polaroid 665 film, and the corresponding negatives were scanned by using a Bio-Rad model 620 densitometer. The data were analyzed using Bio-Rad 1D Analyst software on an IBM PC XT microcomputer, and the ratio of single- to double-stranded cleavage events was determined by assuming that cutting followed a Poisson distribution (15, 16).

Cleavage Site Analysis. Each 50-μl reaction mixture contained 10,000–15,000 counts of 3′- or 5′-end-labeled *Acc* I–*Nde* I restriction fragments from plasmid pUC19 mixed with carrier DNA (sheared salmon testes DNA at 2–5 μg/ml, final concentration; Sigma) and calicheamicin γ' or calicheamicin T in Tris buffer (50 mM Tris·HCl/50 mM NaCl, pH 7.5) containing 10% ethanol. DNA cleavage was initiated by adding 1 mM dithiothreitol, and samples were incubated at 37°C for 30 min. DNA was precipitated, washed, lyophilized, dissolved in sequencing gel-loading buffer (14), and electrophoresed in an 8% polyacrylamide/8.0 M urea slab gel at 1700 V until the bromophenol dye reached the gel bottom. To detect base-sensitive cleavage products, duplicates of some cleavage reactions were dissolved in 100 μl of 0.1 M piperidine and heated at 90°C for 30 min and then lyophilized. After electrophoresis, gels were dried and autoradiographed.

RESULTS

Calicheamicin T Effects Double-Stranded DNA Cleavage. Fig. 1 shows gel electrophoretic patterns for cleavage of supercoiled pBR322 (form I DNA) by calicheamicin γ^1 and calicheamicin T. Much higher concentrations of calicheamicin T than calicheamicin γ^1 are required to effect DNA cleavage. Removing the B-C-D rings decreases the DNA cleavage activity of the drug by ≈3 orders of magnitude, indicating that the B-C-D rings are critical for tight binding. Despite its markedly decreased binding affinity, calicheamicin T shows comparable cleavage activity to double-stranded calicheamicin γ' (Fig. 1, compare lanes 3 and 11). We have quantified the proportions of forms I, II, and III DNA produced as a function of drug concentration. The ratio of single- to double-strand cuts was found to be ≈2:1 for both compounds. Drak *et al.* (13) also report a 2:1 ratio of single- to double-strand cleavage events for calicheamicin γ^1. In contrast, the aglycone of calicheamicin γ^1 effects mainly single-strand breaks (13).

Calicheamicin T Cleaves DNA Nonspecifically. We assessed the site specificity of calicheamicin T by analyzing the cleavage products of an end-labeled *Acc* I–*Nde* I restriction fragment from pUC19. This restriction fragment was chosen to facilitate comparisons with esperamicin D, a similar compound that reportedly cleaves an almost identical (the two fragments differ at the unlabeled end) restriction fragment sequence selectively (10). Figs. 2 and 3 show that calicheamicin T has no significant selectivity. A ladder of cleavage fragments appears on the autoradiogram, and the intensity differs only 2-fold between the darkest and lightest bands. Although some enhancements occur in oligopyrimidine re-

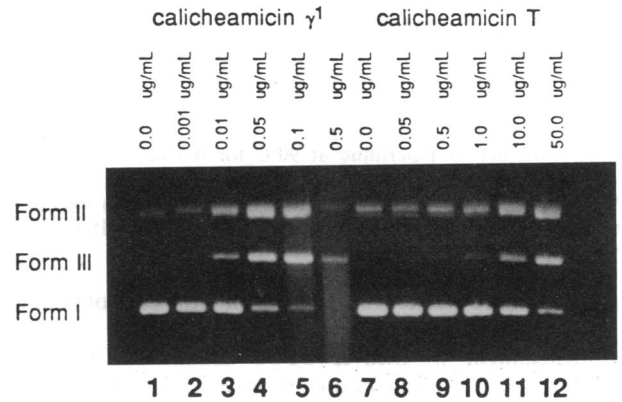


FIG. 1. Agarose gel electrophoretic patterns of pBR322 DNA after treatment with calicheamicins γ^1 and T. One microgram of supercoiled closed circular pBR322 DNA (form I) was incubated with calicheamicin γ^1 or T at the indicated concentrations in a total volume of 20 μl (ethanol/40 mM Tris, 1:9, containing 4 mM EDTA, pH 7.5) in the presence of 1.0 mM dithiothreitol for 1 hr at 37°C and then electrophoresed in a 0.9% agarose gel with added ethidium bromide (0.5 μg/ml) for 6 hr at 80 V.

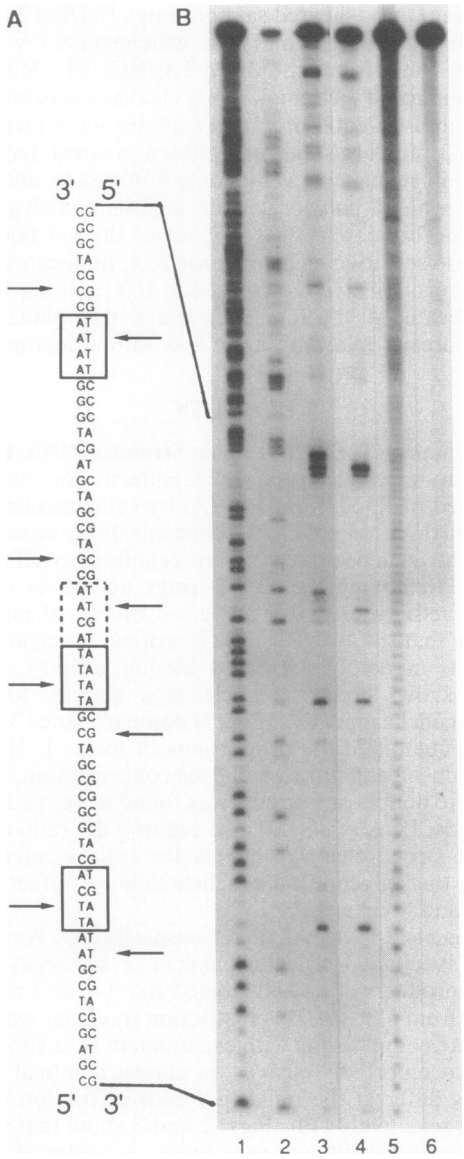


FIG. 2. (*A*) Expanded portion of *Acc* I–*Nde* I fragment. Arrows represent observed cleavage sites; boxes indicate putative contact regions between the carbohydrate tail of calicheamicin γ^1 and DNA. (*B*) Autoradiogram of strand scission by calicheamicins γ^1 and T of a 3′-end-labeled *Acc* I–*Nde* I fragment from pUC19. Lanes: 1 and 2, products of Maxam–Gilbert sequencing reactions (A+G and G, respectively); 3 and 4, products of γ^1 cleavage (incubation conditions: labeled DNA, ethanol/50 mM Tris, 1:9, pH 7.5/calicheamicin γ^1 at 0.25 μg/ml/sheared salmon testes DNA at 5 μg/ml, 25°C). After ethanol precipitation and lyophilization, the reaction of lane 4 was treated with 0.1 M piperidine at 90°C for 0.5 hr to detect C-5′-hydrogen atom abstraction sites (4, 14). Lanes: 5, products of calicheamicin T cleavage (same incubation conditions except the drug was at 200 μg/ml and carrier DNA was at 2 μg/ml); 6, control DNA.

gions, they are so minor that calicheamicin T cannot be called selective.

Calicheamicin γ^1 Cleaves TTTT Tracts. We have also analyzed the cleavage fragments produced by treating the *Acc* I–*Nde* I restriction fragment with calicheamicin γ^1. Calicheamicin γ^1 cleaved quite specifically at four sites. The 4-bp recognition sites that correspond to these cleavage sites were identified on the basis of work by Zein *et al.* (8) and De Voss *et al.* (17). Following Zein *et al.* (8), we identified base-sensitive and base-insensitive cleavage fragments on the 3′-end-labeled restriction fragment (Fig. 2). In accordance with the proposal of Zein *et al.* (8), we assumed that the

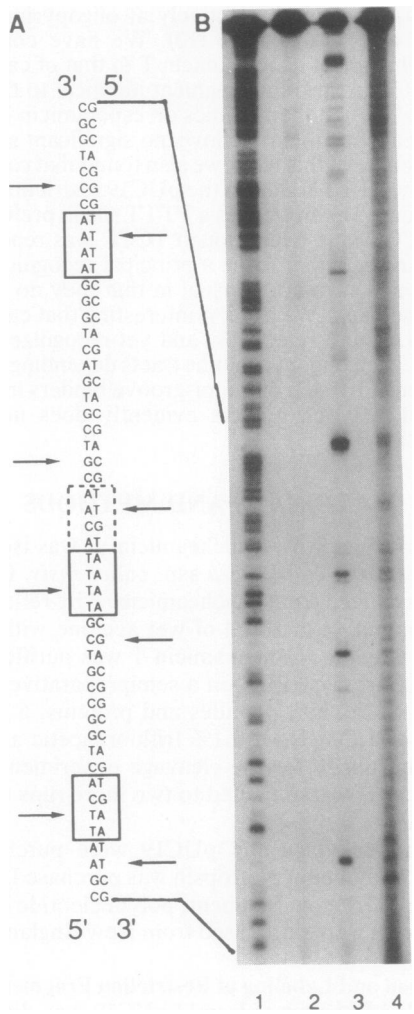


FIG. 3. (*A*) Expanded portion of *Acc* I–*Nde* I fragment. Arrows represent observed cleavage sites; boxes indicate putative regions of contact between the carbohydrate tail of calicheamicin γ^1 and DNA. (*B*) Autoradiogram of strand scission by calicheamicins γ^1 and T of a 5′-end-labeled *Acc* I–*Nde* I fragment from pUC19. Lanes: 1, product of Maxam–Gilbert A+G sequencing reaction; 2, DNA control; 3, products of calicheamicin γ^1 cleavage (incubation conditions: labeled DNA, ethanol/50 mM Tris, 1:9, pH 7.5/calicheamicin γ^1 at 0.25 μg/ml/sheared salmon testes DNA at 5 μg/ml, 25°C); 4, products of calicheamicin T cleavage (same incubation conditions except drug at 300 μg/ml and carrier DNA at 2 μg/ml).

base-sensitive cleavage fragments were produced by abstraction of a C-5′ hydrogen atom from the second nucleotide within the pyrimidine-rich recognition sequence (the product detected before base treatment is apparently the C-5′ aldehyde, which runs more slowly on the gel than the corresponding Maxam–Gilbert marker fragment). De Voss *et al.* (17) have confirmed that a C-5′ hydrogen atom is abstracted from the second nucleotide within the oligopyrimidine strand of the recognition sequence, strengthening these assignments. The fragments whose mobilities are unaltered upon base treatment were assumed to be produced by cleavage of the purine-rich complementary strand, where a different hydrogen atom is apparently abstracted (cleavage is staggered 3 bp in the 3′ direction, so that it is actually in the flanking sequence). On this basis, the four recognition sites were tentatively identified as follows (5′ → 3′, giving only the oligopyrimidine strand): TTCA (strong), TTTT (strong), TTGT (weak), and TTTT (strong). The fragments produced by cleavage of the corresponding 5′-end-labeled restriction fragment confirmed the assignments (Fig. 3).

There is no TCCT site in the readable portion of the *Acc* I–*Nde* I pUC19 restriction fragment so we could not compare the efficiency of cleavage at TTTT tracts to that at the reported optimum cleavage site. There is, however, a TCCC site to the 3′ side of one of the TTTT sites (it actually overlaps by 1 bp). TCCC was reported to be a principal cleavage site for calicheamicin γ^1 by Zein *et al.* (8). In this restriction fragment, the TTTT site is cleaved *in preference* to the adjacent TCCC site. If cleavage selectivity reflects binding selectivity (and we think it does, *vide infra*), then calicheamicin must bind more strongly to the A·T-rich homopyrimidine sequence than to the G·C-rich homopyrimidine sequence *in this case*. That TCCC sites are cleaved strongly in other restriction fragments indicates that flanking sequences significantly affect binding (8).

DISCUSSION

The A-E Disaccharide Facilitates Binding of the Aglycone in the Minor Groove. Drak *et al.* (13) have shown that the calicheamicin aglycone cleaves DNA in a predominantly single-stranded manner (single-stranded cuts/double-stranded cuts, 30:1), implying that the aglycone cannot bind efficiently in the minor groove. As shown above, calicheamicin T cleaves DNA in a double-stranded manner (single-stranded cuts/double-stranded cuts, 2:1). Calicheamicin T thus binds to DNA so it can reach both DNA backbone strands upon cyclization. We have evidence that calicheamicin T binds in the minor groove: netropsin, a molecule known to bind tightly in the minor groove, protects several A·T-rich regions of DNA from cleavage by calicheamicin T (data not shown). Thus, the ability to cleave DNA in a double-stranded manner like the parent molecule probably reflects an ability to bind in the minor groove in an orientation roughly similar to that of calicheamicin itself.

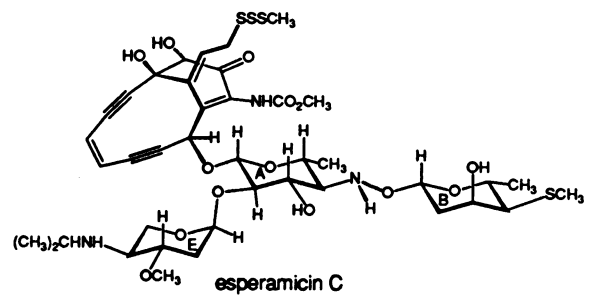
Cyclization Kinetics Do Not Influence Cleavage Selectivity in Calicheamicin T. De Voss *et al.* (18) have pointed out that the cleavage selectivity of calicheamicin γ^1 could arise from either preferential binding to the recognition site or an accelerated rate of Bergman cyclization induced by the environment at the recognition site or some combination of both (19, 20). Our results show that calicheamicin T binds in the minor groove and yet cleaves all nucleotides comparably. Therefore, the cyclization rates to form the diradical do not differ significantly *as detected by cleavage selectivity*. The Bergman cyclization in calicheamicin T appears to be insensitive to local variations in groove conformation and sequence. We believe that these results imply that cyclization kinetics do not play the primary role in determining cleavage selectivity of calicheamicin γ^1 *unless* interactions between the carbohydrate tail and DNA at sites that are not thermodynamically preferred (otherwise thermodynamics is the primary determinant of site-selective cleavage) somehow facilitate the rearrangement. This seems improbable. Because the cyclization rate of the diyne-ene appears rather insensitive to environmental variations within the minor groove, we think the cleavage selectivity seen with calicheamicin γ^1 is primarily due to thermodynamic binding selectivity.

Calicheamicin γ^1 Is Not Selective for G·C-Containing Sequences. We were surprised to find that calicheamicin γ^1 selectively cleaves TTTT sites [S. Mah, C. Townsend, and T. Tullius (personal communication) have also observed strong cutting at sites that lack G·C base pairs]. Crystallographic data, NMR results, and experiments with chemical probes indicate that DNA sequences containing four or more sequential A·T base pairs have very different conformational properties from DNA sequences including G·C base pairs (21, 27). Moreover, guanines have amino groups that protrude in the minor groove, and the microenvironment around a G·C base pair differs both sterically and in hydrogen-bonding potential from that around an A·T base pair. Most site-selective minor groove binders are classified by whether they bind to A·T-rich sequences like netropsin and distamycin (28, 29) or to sequences including G·C base pairs like chromomycin (30). In the original cleavage paper by Zein *et al.* (8) all reported cleavage sites contained G·C base pairs. It has apparently been assumed that calicheamicin is selective for oligopyrimidine sequences including G·C base pairs. Our results show that there is no requirement for even a single G·C base pair in the recognition sequence. The only feature shared by all the reported cleavage sites is that they include at least three pyrimidines.

The cleavage evidence obtained to date suggests that calicheamicin γ^1 recognizes DNA conformation and not sequence *per se* (31–36). However, the conformation recognized is not a unique property of the sequence TCCT and a limited number of closely related sequences. Calicheamicin recognizes many oligopyrimidine sequences. Given the diversity of sequences recognized, calicheamicin γ^1 probably does not sense inherent groove conformation (i.e., intrinsic local DNA secondary structure) in all cases. We suggest that there is some degree of "induced fit" in the recognition event (37, 38). This feature may be important in determining the site selectivity of calicheamicin as well (13). Structural information on calicheamicin binding to TTTT, TCCT, and perhaps other sequences will be necessary to fully understand the recognition process.

The Role of the Aglycone in Specificity Remains Unclear. Drak *et al.* (13) have shown that the calicheamicin aglycone has no cleavage specificity. They have concluded that the carbohydrate-aryl tail determines cleavage specificity of calicheamicin γ^1. Results on calicheamicin T support the idea that the carbohydrate-aryl tail is critical for the cleavage selectivity of calicheamicin γ^1. However, it is not yet known whether the aglycone contributes to specificity in the intact drug. The binding specificity of a molecule is usually not just a sum of specificities of the individual components; nor is binding energy generally a sum of binding energies of the individual components (39). Experiments on a limited set of truncated deriveatives can be misleading since the "missing" specificity cannot simply be attributed to the missing components. In calicheamicin little flexibility exists in the linkage connecting the oligosaccharide-aryl tail to the aglycone (40). It would be surprising if the aglycone had no effect on the "inherent" specificity of the oligosaccharide-aryl tail. However, whether the aglycone plays a role in the specificity of the intact drug could be tested by determining the specificity of the oligosaccharide alone.

Role of the Thiobenzoate Ring. From comparisons of cleavage selectivity of esperamicin A_1 and C with calicheamicin



esperamicin C

γ^1, Hawley *et al.* (11) and Drak *et al.* (13) proposed that the thiobenzoate ring plays a primary role in site selectivity. Although the thiobenzoate ring is crucial for the high cleavage selectivity obtained with calicheamicin γ^1, we suggest that its relative importance may have been overstated. The cleavage evidence shows that calicheamicin cleaves at both A·T tracts and G·C-rich sequences. The thiobenzoate ring is

not recognizing specific base pairs, nor is it selecting for inherent groove width [it has been proposed (41) that the piperazine ring in Hoechst 33258 is a weak G·C recognition element because it requires the extra groove width associated with G·C base pairs to fit into the minor groove]. Moreover, the thiobenzoate ring is only large enough to contact a portion of the recognition sequence. Imagining a mechanism whereby this ring could confer significant binding site selectivity on its own is difficult. Its orientation and location within calicheamicin must be critical for its effect. The danger in using a limited set of truncated derivatives to study the role that a particular molecular component plays in specificity is that it is easy to overemphasize individual contributions—that is, to attribute to one subunit properties that depend on other parts of the molecule as well. Again, the whole may be greater than the sum of the parts. It will probably be necessary to modify the thiobenzoate ring without removing it entirely to shed more light on its role in the recognition process.

Summary. We have shown that a truncated derivative of calicheamicin cleaves DNA in a double-stranded manner. Because Drak *et al.* (13) showed that the aglycone effects primarily single-stranded cleavage, our results indicate that the A-E disaccharide is sufficient to help the aglycone bind in the minor groove. We have further shown that this truncated derivative cleaves DNA nonspecifically, suggesting that in the intact drug, cleavage selectivity reflects binding selectivity rather than cyclization kinetics. We have also found that calicheamicin recognizes a wider range of sequences than originally thought and that G·C base pairs are not needed in the recognition sequence. In fact, calicheamicin recognizes *both* A·T tracts and G·C-rich oligopyrimidine sequences with a high degree of selectivity, making it an unusual minor-groove binder.

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